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# Generation of islet-like cells from mouse gall bladder by direct *ex vivo* reprogramming

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**Abstract** Cell replacement is an emerging therapy for type 1 diabetes. Pluripotent stem cells have received a lot of attention as a potential source of transplantable β-cells, but their ability to form teratomas poses significant risks. Here, we evaluated the potential of primary mouse gall bladder epithelial cells (GBCs) as targets for *ex vivo* genetic reprogramming to the β-cell fate. Conditions for robust expansion and genetic transduction of primary GBCs by adenoviral vectors were developed. Using a GFP reporter for insulin, conditions for reprogramming were then optimized. Global expression analysis by RNA-sequencing was used to quantitatively compare reprogrammed GBCs (rGBCs) to true β-cells, revealing both similarities and differences. Adenoviral-mediated expression of *NEUROG3*, *Pdx1*, and *MafA* in GBCs resulted in robust induction of pancreatic endocrine genes, including *Ins1*, *Ins2*, *Neurod1*, *Nkx2-2* and *Isl1*. Furthermore, expression of GBC-specific genes was repressed, including *Sox17* and *Hes1*. Reprogramming was also enhanced by addition of retinoic acid and inhibition of Notch signaling. Importantly, rGBCs were able to engraft long term *in vivo* and remained insulin-positive for 15 weeks. We conclude that GBCs are a viable source for autologous cell replacement in diabetes, but that complete reprogramming will require further manipulations.


*Abbreviations:* PSCs, pluripotent stem cells; GBC, gall bladder cell; RA, retinoic acid; DBZ, dibenzazepine; GSIS, glucose-stimulated insulin secretion; qPCR, quantitative PCR; RT-PCR, reverse transcription PCR; FACS, fluorescence-activated cell sorting.

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## Introduction

The initial success of the Edmonton protocol highlighted the potential of cell replacement therapy in type 1 diabetes (Shapiro et al., 2000). However, wider application of this approach is severely limited by the shortage of transplantable pancreatic β-cells (de Kort et al., 2011). In addition, the transplantation of cadaveric islets requires life-long immune suppression (Ricordi and Strom, 2004). Ideally, therefore, a source of transplantable β-cells would be both autologous and abundant. Because of their *in vitro* growth capacity, pluripotent stem cells (PSCs) are an attractive potential source of transplantable β-cells. While significant progress has been made, the generation of true β-cells *in vitro* has remained elusive thus far (Alipio et al., 2010; D'Amour et al., 2006; Nostro et al., 2011). In addition, PSCs are capable of forming teratomas and represent an unknown risk in terms of tumorigenesis (Fujikawa et al., 2005; Kroon et al., 2008).

Direct genetic reprogramming of postnatal primary cells by forced expression of key developmental transcription factors has emerged as an alternative to *in vitro* differentiation of PSCs. This strategy has been used successfully to produce functional cells, including neurons, hepatocyte-like cells and cardiomyocytes from fibroblasts (Huang et al., 2011; Ieda et al., 2010; Vierbuchen et al., 2010). Similarly, *in vivo* reprogramming of hepatic cells by expressing different pancreatic transcription factors, including *Neurog3* and *Pdx1*, was able to restore euglycemia in hyperglycemic mice (Ferber et al., 2000; Wang et al., 2007; Vijay Yechoor et al., 2009). Additionally, *in vivo* reprogramming of exocrine acinar cells into insulin-positive cells by expression of *Neurog3*, *Pdx1* and *MafA* was also able to reverse hyperglycemia in mice (Zhou et al., 2008). Furthermore, overexpression of *Neurog3* and *Pdx1* has been shown to enhance pancreatic differentiation of embryonic stem cells (Kubo et al., 2011). Other cell types have also been tested for amenability to reprogramming towards the β-cell fate, including adipose tissue-derived stem cells, placenta-derived multipotent stem cells, hepatocytes, intrahepatic biliary epithelial cells and gall bladder cells (Chandra et al., 2011; Chiou et al., 2011; Coad et al., 2009; Motoyama et al., 2009; Nagaya et al., 2009; Shigeru et al., 2007).

The extrahepatic biliary tissue, including the gallbladder, is a particularly appealing source of cells for reprogramming to the pancreatic fate. The extrahepatobiliary system shares a common developmental origin with the ventral pancreas, from a cell termed the pancreatobiliary progenitor (Spence et al., 2009). Segregation of these distinct lineages is partly regulated by the Notch effector *Hes1*. Recently it was demonstrated that inhibition of Hes1 in cultured gall bladder cells (GBCs) was sufficient to induce some insulin expression (Coad et al., 2009). While this work highlighted the potential of GBCs as a source of transplantable β-cells, the full spectrum of β-cell expressed genes or the *in vivo* functionality of these cells was not determined. Moreover, the cells showed only limited proliferative potential under the culture conditions used. For GBCs to be a viable substrate of future β-cell replacement therapies, they would have to be robustly expandable (Yechoor and Chan, 2010). Therefore, the true utility of GBCs as a source of transplantable β-cells remains unknown.

In this study, we investigated if mouse GBCs significantly expanded *in vitro* can still be reprogrammed towards the β-cell fate by using a combination of positive instructive signals as well as Notch inhibition. GBCs were transduced with adenoviruses expressing the transcription factors *NEUROG3*, *Pdx1* and *MafA* and treated with retinoic acid and Notch inhibitors, resulting in their differentiation into islet-like cells. Reprogrammed cells had the ability to engraft, survive and remain insulin-positive up to 15 weeks post-transplantation. However, there were also differences between the reprogrammed GBCs and true β-cells. Our findings confirm that the gall bladder represents a promising source of autologous reprogrammable cells for the treatment of type 1 diabetes mellitus.

## Materials & methods

### Mouse gall bladder cell isolation and culture

Gall bladders from C57Bl6/6J-MIP-GFP male and female mice between the ages of 4 and 8 weeks were removed by a surgical incision and bile released by making a single cut in the wall. Gall bladders were rinsed twice in DPBS (Life Technologies, Grand Island, Ca) and then cut into several pieces. This material was then incubated at 37 °C with 0.25% Trypsin/EDTA (Life Technologies, Grand Island, Ca) for 45 min to obtain a cell suspension. Cells were cultured using a modified protocol to that previously described (Manohar et al., 2011). Briefly, cells were plated on a 70–80% confluent irradiated LA7 rat epithelial feeder layer that had been previously irradiated at 60 Gy. Cells were cultured in DMEM/F12 (Life Technologies, Grand Island, Ca) supplemented with 0.5% FBS (Thermo Fisher Scientific, Cambridge, MA), 1% insulin–transferrin–selenium (Roche, Indianapolis, IN), 15 mM HEPES (Thermo Fisher Scientific, Cambridge, MA) and antimicrobials (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Amphotericin B; Cellgro, Manassas, VA) in a 37 °C incubator with 5% $CO_2$. Media was changed every two to three days. When GBCs were 70–90% confluent, they were passaged by incubation with 0.05% trypsin/EDTA (Life Technologies, Grand Island, Ca) at 37 °C, followed by incubation with DNaseI at 37 °C for 10 min to obtain a single cell suspension.

### Fibroblast culture

For initiating fibroblast cultures, mouse tail-tips from euthanized C57Bl6/6J-MIP-GFP mice were washed with DPBS, cut into several pieces, and digested with 0.25% Trypsin/EDTA for 60 min at 37 °C with regular mixing. Upon inactivation of trypsin by addition of serum, the tissue mix was spun at 1000 rpm for 5 min, resuspended in DMEM supplemented with 15% FBS and antimicrobials, followed by plating in a 37 °C incubator with 5% $CO_2$. Media was changed every two to three days.

### Adenovirus transduction of GBCs

Each E1-deleted adenovirus (serotype 5) consisted of the full-length cDNA (human *NEUROG*, rat *Pdx1*, mouse *MafA*; *NEUROG3* and *Pdx1* provided by Michael German, University of California at San Francisco; *MafA* provided by Roland Stein, Vanderbilt University Medical Center) driven by the

CMV promoter. For additional amplification, each virus was expanded in HEK293 cells and purified using the FastTrap Purification Kit (Millipore, Billerica, MA) per the manufacturer's protocol. Adenovirus titers were calculated based on spectrometry. Adenoviruses expressing *NEUROG3* (MOI 1000), *Pdx1* (MOI 500), and *MafA* (MOI 500) were incubated in 100 μg/ml DEAE-Dextran (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature with regular mixing prior to addition to the cell media for a final concentration of 10 μg/ml DEAE-Dextran. Media was changed 20–24 h post transduction.

## GBC reprogramming

GBC cultures were grown to ~70% confluency. On this day, day 0, cells were transduced with the adenovirus/DEAE-Dextran mix. 20–24 h later, the media was changed to include 2 μM retinoic acid (Sigma-Aldrich, St. Louis, MO) and 1% DMSO (Thermo Fisher Scientific, Cambridge, MA). Twenty four hours later, the media was changed to include 250 nM of the γ-secretase inhibitor dibenzazepine (DBZ; EMD Chemicals, Darmstadt, Germany) and 1% DMSO. All cells, attached and in suspension, were kept by spinning the media to pellet all cells prior to addition of new media.

## Flow cytometry and FACS

For antibody labeling, GBCs were dissociated into single cells using a non-trypsin method (Cell dissociation buffer; Gibco, Grand Island, CA). Dissociated cells were resuspended in DMEM, supplemented with 2% FBS and 0.25 mg/ml DNaseI (Sigma-Aldrich, St. Louis, MO). Propidium iodide staining was used to label dead cells for exclusion. The forward scatter (FSC):pulse width gating excluded cell doublets from sorts, as previously described (Dorrell et al., 2008). Cells were analyzed with a FACScalibur or sorted by an inFluxV-GS (BD Biosciences, San Jose, CA for both) at 15 psi using a 100 μm nozzle. Data were analyzed using FlowJo (Treestar, Ashland, OR).

## RNA isolation and qRT-PCR

For RNA isolation, cells were either directly FACS-sorted into Trizol Liquid Sample (Life Technologies, Grand Island, CA) or trypsinized and pelleted by centrifugation prior to cell lysis with Trizol. RNA was purified using RNeasy (Qiagen, Valencia, CA) per the manufacturer's protocol. First strand cDNA synthesis was completed using MMLV reverse transcriptase and random oligonucleotide primers (Life Technologies, Grand Island, CA). Relative mRNA expression levels were determined by qRT-PCR using a BioRad iCycler with a single color MyiQ detection system. All reactions were performed with Platinum Taq DNA Polymerase (Life Technologies, Grand Island, CA) and SYBR Green using 45 cycles of 95 °C for 15 s, 68 °C for 20 s, and 72 °C for 20 s. The full list of mouse-specific primers is given in Table S4. Results were analyzed using gene expression relative to control *Alas1* or *Actb* gene expression ($\Delta\Delta C_T$). qPCR data were expressed as mean fold change ($2^{\Delta\Delta CT}$) ± 95% confidence.

## Immunohistochemistry and immunofluorescent imaging

For cytospin imaging, either reprogrammed GFP+ cells or non-adenovirus transduced control cells were spun at 1000 rpm for 5 min onto Superfrost Plus slides (Thermo Fisher Scientific, Cambridge, MA). Cells were fixed in either 4% paraformaldehyde or 90% methanol at 4 °C for 10 min. Prior to labeling, cells were blocked in 5% BSA for 60 min at 23 °C. Primary labeling was performed overnight at 4 °C in PBS supplemented with 2% BSA and 0.05% Triton-X using rabbit polyclonal antibodies against insulin (H-86; Santa Cruz Biotechnology, Santa Cruz, CA), C-peptide (BCBC collection #1042), Neurod1 (16508; Abcam, Cambridge, MA) and Somatostatin (A0566; DAKO, Carpinteria, CA). Secondary labeling was performed for 60 min at 23 °C in PBS supplemented with 2% BSA and 0.05% Triton-X with a 1:200 dilution of Alexa 555-conjugated goat anti-rabbit IgG (Cell Signaling, Danvers, MA). Nuclei were stained using Hoechst 33342 (Sigma-Aldrich, St. Louis, MO). For IHC analysis, formalin-fixed paraffin-embedded kidneys were sectioned and labeled with a primary antibody against insulin (H-86; Santa Cruz Biotechnology, Santa Cruz, CA) and detected using previously described methods (Overturf et al., 1996).

## Enzyme-linked immunosorbent assay

Control and rGBCs were harvested at the indicated time points. Cells were washed twice in Krebs Ringer Buffer supplemented with 2.8 mM glucose (KRB-2.8) and incubated at 37 °C for 90 min in KRB. Following two further washes in KRB-2.8, cells were incubated in either KRB-2.8 or Krebs Ringer Buffer supplemented with 16.7 mM glucose (KRB-16.7) for 60 min at 37 °C. Cells were spun at 1200 rpm for 5 min and the media collected and stored at −80 °C. Insulin quantitative analyses were performed as per the manufacturer's instructions (Insulin Ultrasensitive ELISA, Alpco, Salem, NH).

## Cell transplantation

GBCs were expanded to 70% confluency prior to reprogramming at the indicated passage number (Table S3). Cells were transduced with the three adenoviruses expressing *NEUROG3*, *Pdx1* and *MafA* with or without retinoic acid as described in Table S1. One to three days later, cells were harvested and shipped on ice to the University of Massachusetts Medical School (Worcester, MA) for transplantation on the following day. Cell viability was determined by propidium iodide staining after shipping and prior to transplantation. $20 \times 10^6$ unsorted cells were transplanted under the renal capsule of diabetic NOD.Cg-*Rag1*$^{tm1Mom}$ *IL2rg*$^{tm1Wjl}$*Ins2*$^{Akita}$ (NRG-Akita) and NOD.*Cg-Prkdc*$^{scid}$*Il2rg*$^{tm1Wjl}$/*SzJ Ins2*$^{Akita}$ (NSG-Akita) mice, as previously described (Brehm et al., 2010). Non-fasting blood glucose levels were monitored following transplantation by blood glucose measurements with an ACCUCHEK active glucometer (Hoffman-LaRoche, Basel, Switzerland). The Institutional Animal Care and Use Committee approved all animal studies.

### RNA-SEQ analysis

Libraries were single-read sequenced to 100 bp on an Illumina hiSeq2000. Reads coming from ribosomal RNA and repeats were filtered out by aligning 50 bp of all reads to mouse ribosomal sequence and the human contents of Repbase (release 14.10) (Jurka et al., 2005). The remaining reads where processed with RUM (Grant et al., 2011) using the default parameters to align reads to genome (mm9) and gene models and to produce transcript-level quantification in reads. Expression levels (in reads) were log2 transformed and then normalized using quantile normalization (R limma). After unclogging, data were converted to reads and averaged between replicates. Differential expression was determined using the Fisher exact test and Benjamini–Hochberg correction for multiple testing on genes with at least a two-fold difference between conditions. Gene lists from either the up- or down-regulated portion of each comparison were analyzed using Ingenuity Core Analysis (Ingenuity Systems, www.ingenuity.com). The RNA-Seq data was submitted to the European Bioinformatics Institute ArrayExpress (http://www.ebi.ac.uk/arrayexpress/).

### Statistical analysis

Statistical analyses were conducted with GraphPad Prism software v.4.0 or Microsoft Excel. Experimental differences were evaluated by student two-tailed *t*-test assuming equal variance. $P$ values $< 0.05$ were considered statistically significant.

## Results

### Expansion and transduction of mouse gall bladder cells

Cell replacement therapy for type 1 diabetes requires large numbers of functional cells. Therefore, the expandability of primary GBCs had to be demonstrated in order for the adult gall bladder to be a viable alternative to pluripotent stem cells (PSCs), which can be readily grown *in vitro*. A modified protocol first reported by Manohar et al. was used to isolate and expand mouse GBCs (Manohar et al., 2011). GBCs were cultured on a feeder layer of irradiated LA7 rat epithelial cells (Dulbecco et al., 1979) that allowed their robust expansion. Upon initial harvest a typical mouse gall bladder yielded approximately 200–400,000 cells that showed unlimited ability to replicate and expand, normally generating hundreds of millions of cells by passage 3 (Figs. 1A–C). Expanded cells also maintained their epithelial characteristics and there was no evidence of epithelial–mesenchymal transition based on expression of known mesenchymal markers (Kalluri and Weinberg, 2009), including *Acta2*, *Col1a1*, *Ddr2*, *S100a4* and *Vim* (Table S1). Next, the suitability of adenoviral vectors for the introduction of transgenes in expanded GBCs was assessed. Less than 10% of the cells were positive for the marker transgene GFP when using standard transduction protocols (Figs. 1D, E). DEAE-dextran has previously been shown to enhance the transduction of other epithelial cells by adenoviral vectors (Gregory et al., 2003; Kaplan et al., 1998). Concentrations greater than 10 μg/ml allowed transduction of more than 50% of the GBCs (Figs. 1D, F). Although higher concentrations of DEAE-Dextran gave even greater transduction efficiencies, they were also associated with increased cell mortality (data not shown). Therefore, for all the reprogramming experiments, a final DEAE-Dextran concentration of 10 μg/ml was used for adenovirus transduction of GBCs.

### NEUROG3, Pdx1 and MafA are required for optimal expression of both *Ins1* and *Ins2*

In order to determine the optimal combination of reprogramming transcription factors, a mouse reporter strain (MIP-GFP Hara et al., 2003) in which the mouse *Ins1* promoter drives expression of GFP was utilized. Multiple pancreatic developmental transcription factors were tested singly and in combinations, including Pdx1 (Jonsson et al., 1994), NEUROG3 (Apelqvist et al., 1999), MafA (Nishimura et al., 2006), and Nkx6-1 (Sander et al., 2000). In control GBCs, no GFP expression was detected, indicating absence of transcriptional activity from the *Ins1* promoter (Fig. 2). Previous work has shown that forced expression of *Neurog3* or *Pdx1* is sufficient to differentiate liver cells towards a pancreatic fate *in vivo* (Ferber et al., 2000; Wang et al., 2007). Although expression of these two factors together induced low levels of GFP expression, adenoviral-mediated expression of *MafA*, together with *NEUROG3* and *Pdx1*, was required for optimal GFP expression in GBCs *in vitro* (Fig. 2A). Additional transduction of GBCs with Nkx 6-1 as a fourth factor did not yield increased reprogramming (data not shown). A second reporter strain was also generated by breeding a mouse expressing Cre recombinase under control of the rat *Ins2* promoter with a dual reporter Tomato-RFP/GFP mouse in which expression of GFP is only detected in cells in which a Cre-mediated recombination event has occurred (Muzumdar et al., 2007; Postic et al., 1999). Control cultured GBCs were always GFP negative, whereas GBCs reprogrammed with *NEUROG3*, *Pdx1* and *MafA* (NPM) activated the *Ins2* promoter (Fig. S1). These results indicated that both insulin promoters were active in reprogrammed cells. For all subsequent experiments, GBCs expanded from MIP-GFP mice (*Ins1* promoter) were used.

### Retinoic acid and Notch inhibition enhance reprogramming of GBCs

Small molecules have previously been successively used for reprogramming PSCs towards a pancreatic lineage (Kroon et al., 2008; Nostro et al., 2011). The effect of retinoic acid (RA) on reprogramming was first tested. By including RA in the reprogramming media at a concentration of 2 μM, there was a significant 2.2 fold increase in the percentage of GFP+ GBCs reprogrammed with *NEUROG3*, *Pdx1* and *MafA* (NPM), compared to GBCs reprogrammed with NPM alone (Fig. 2A). The effect of inhibiting Notch signaling by using the gamma secretase inhibitor dibenzazepine (DBZ) was examined next (van Es et al., 2005). Inhibition of Notch signaling at day 2 of reprogramming caused a further significant increase in GFP+ reprogrammed cells (Fig. 2A). RA or DBZ in the absence of

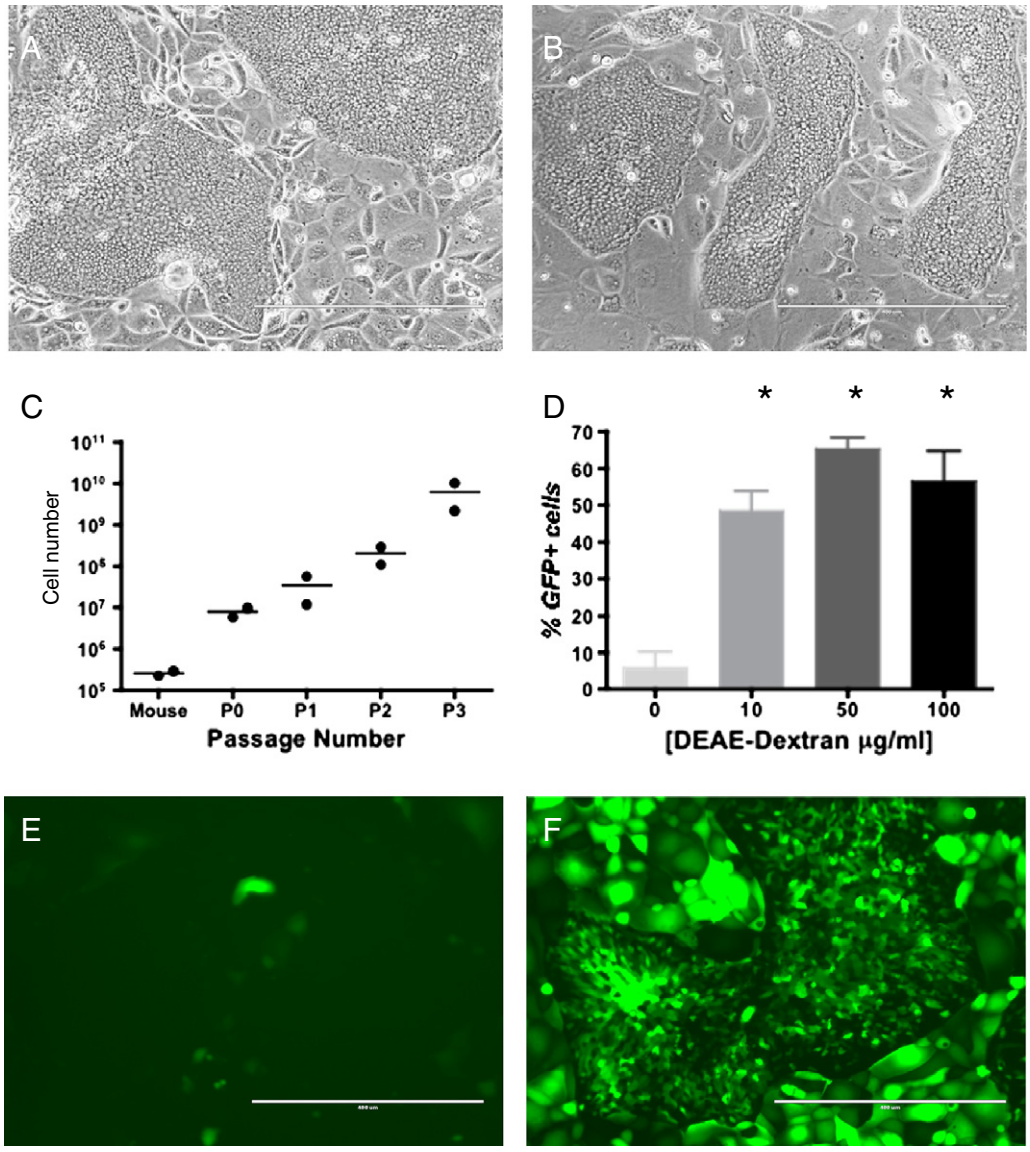


**Figure 1** Expansion and adenovirus transduction of GBCs. (A, B) Phase contrast images of passage 0 (A) and passage 3 (B) typical GBC colonies. (C) GBCs were robustly expanded, generating hundreds of millions of cells by passage 3 (P3). The data shown is for two independent gall bladders. (D) Without any DEAE-Dextran, only ~5% of cells were transduced by adenovirus expressing GFP at 500 MOI. However, with increasing concentrations of DEAE-Dextran, there was a significant increase in the number of GBCs transduced (*$p < 0.05$; N = 3). (E, F) Fluorescent images showing (E) few GBCs GFP+ without DEAE-Dextran addition, whereas at 10 µg/ml (F), ~70% of the GBCs were GFP+. Scale bar for all images is 400 µm.

NPM did not cause any GFP+ cells to appear. The optimized reprogramming timeline is shown in Fig. S2 and a representative FACS plot of GFP expression after reprogramming is shown in Fig. 2B. As the cells were reprogrammed towards the pancreatic fate, the morphology of the cells changed and the GBCs lost their distinctive tight colony structure, eventually loosing adherence and floating in suspension in the culture. We next analyzed the expression of GFP over a period of 12 days after reprogramming in two different gall bladder samples (Fig. S3). The first GFP+ cells appeared within 48 h after reprogramming was begun, with the maximum number of GFP+ cells detected 24 h later. After this time, the percentage of GFP+ cells decreased, coinciding with increased cell death under the culture conditions used (data not shown).

## GBCs are rapidly reprogrammed towards the β-cell-fate

Four days after reprogramming, reprogrammed GFP+ GBCs were analyzed by flow cytometry and FACS-sorted for RNA and protein analyses. Adenovirus GFP-only transduced GBCs were used as the control. Using RNA-Seq, true reprogramming of these GBCs was evident by induction of genes involved in several aspects of β-cell function (Table 1) including

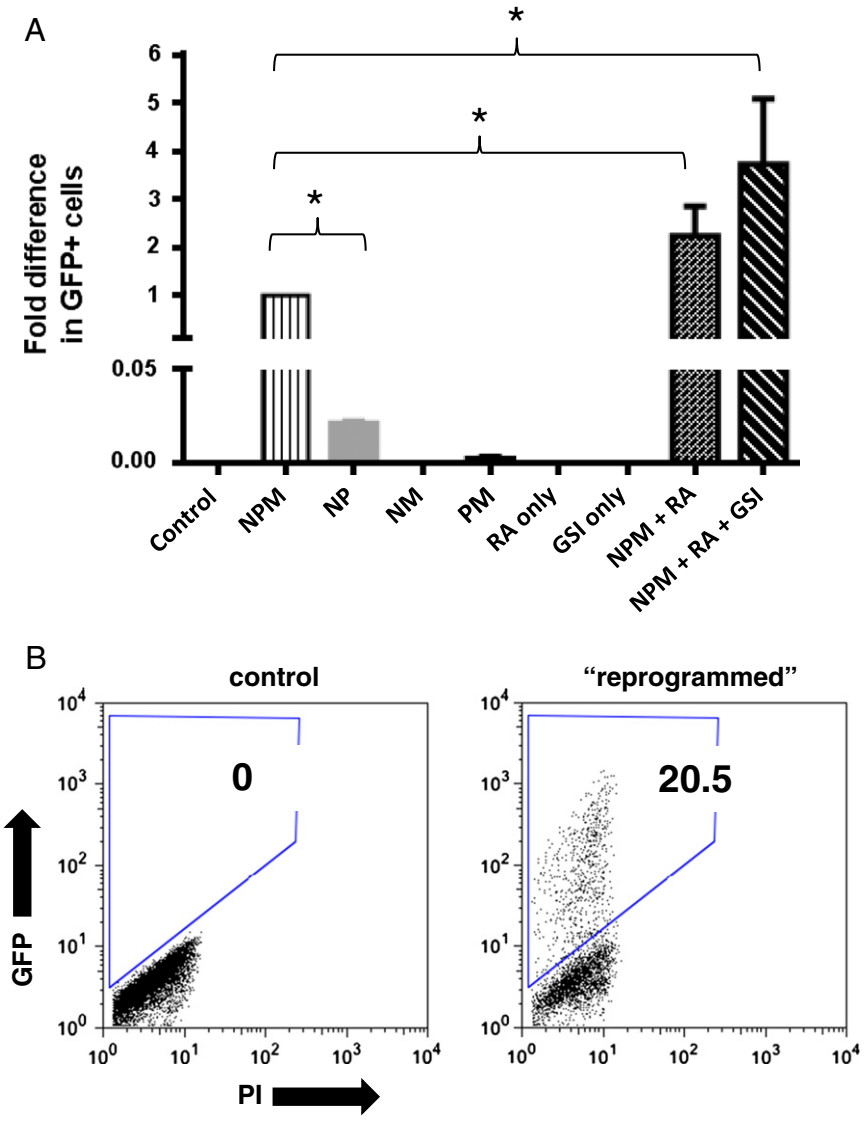


**Figure 2** Optimization of GBC reprogramming. (A) Comparison of the percentage of GFP (=insulin1) expressing GBCs after reprogramming. GFP+ percentage after reprogramming with the three transcription factors — *NEUROG3*, *Pdx1* and *MafA* (NPM) was arbitrarily set to 1. The combination of all three factors yielded significantly higher reprogramming rates compared to any combination of two factors (*$p < 0.05$; N = 3). RA increased the percentage of GFP+ GBCs in cultures by approximately 2.2 fold (*$p < 0.05$; N = 3). Addition of DBZ to the reprogramming mix on day 2 also increased the number of GFP+ GBCs. Addition of RA or GSI alone had no effect on reprogramming. (B) GBCs from MIP-GFP mice were GFP-negative by flow cytometry, indicating no activity of the *Ins1* promoter. Three days after reprogramming, 20.5% of total GBCs in the plate became GFP+ using the optimized protocol depicted in Fig. S2.

proinsulin production (*Ins1*, *Ins2*), insulin processing (*Pcsk1*, *Pcsk2*, *Cpe*), transcription factors (*Nkx2-2*, *Neurod1*, *Isl1*), glucose metabolism (*Gck*), ATP-sensitive $K^+$ channels (*Kcnj11*), calcium channels (*Cacna1a*) and insulin secretion (*Chga*, *Scg3*). RNA-Seq data was validated for several genes by qRT-PCR (Figs. S4A, B). In accordance with the gene expression data, rGBCs also expressed the proteins for insulin, c-peptide, and Neurod1 (Fig. 3). In fact, all GFP+ FACS-sorted cells were positive for these three proteins, indicating that the MIP-GFP reporter system was an excellent read-out for GBC-derived islet-like cells (Fig. 3). These results clearly demonstrated the activation of many genes specific for pancreatic β-cells. However, complete reprogramming involves not only the activation of desired genes, but also the silencing of genes specific for the cell type of origin. Within three days post reprogramming, GFP+ GBCs showed significant decreases in expression of several genes that are normally expressed in this cell type (Table S2). In addition, the pancreatobiliary transcription factors *Sox17* and *Hes1*, required for normal gall bladder development and homeostasis, were also reduced. The RNA-Seq data for these genes was also validated by PCR (Fig. S4B).

## The pancreatic endocrine phenotype of rGBCs is polyhormonal and non-glucose-responsive

Pancreatic endocrine cells produced by *in vitro* differentiation of PSCs are mostly polyhormonal (D'Amour et al., 2006;

**Table 1** Expression levels of β-cell-related genes in rGBCs relative to control GBCs. FC, fold change.

| Gene | Transcript | Description | FC | p value |
|---|---|---|---|---|
| *Ins1* | NM_008386 | Insulin I | 4837.3 | 4.9E−04 |
| *Ins2* | NM_001185083 | Insulin II | 7082.3 | 5.5E−06 |
| *Pcsk1* | NM_013628 | Proprotein convertase subtilisin/kexin type 1 | 16.6 | 1.9E−01 |
| *Pcsk2* | NM_008792 | Proprotein convertase subtilisin/kexin type 2 | 687.4 | 8.6E−02 |
| *Cpe* | NM_013494 | Carboxypeptidase E | 11.0 | 5.2E−03 |
| *Nkx2-2* | NM_010919 | NK2 transcription factor related, locus 2 | 11,625.5 | 9.9E−04 |
| *Neurod1* | NM_010894 | Neurogenic differentiation 1 | 40,063.5 | 2.3E−04 |
| *Isl1* | NM_021459 | ISL1 transcription factor, LIM/homeodomain | 2749.6 | 1.6E−03 |
| *Pdx1* | NM_008814 | Pancreatic and duodenal homeobox 1 | 8.5 | 7.1E−03 |
| *Gck* | NM_010292 | Glucokinase | 28.3 | 4.1E−02 |
| *Kcnj11* | NM_010602 | Potassium inwardly rectifying channel, subfamily J, member 11 | 10,333.2 | 3.1E−04 |
| *Cacna1a* | NM_007578 | Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit | 24.7 | 1.5E−04 |
| *Slc2a2* | NM_031197 | Solute carrier family 2 (facilitated glucose transporter), member 2 | 60.5 | 5.1E−03 |
| *Chga* | NM_007693 | Chromogranin A | 2157.3 | 1.0E−03 |
| *Scg3* | NM_001164790 | Secretogranin III | 52,681.2 | 1.7E−05 |

Nostro et al., 2011). In order to determine whether genetic reprogramming of GBCs resulted in the emergence of pancreatic cell types other than β-cells, qPCR analysis of mRNA expression was done initially on non-FACS sorted cells. In addition to insulin, other endocrine hormones were also produced, including *Sst*, *Ppy*, and *Ghrl* gene transcripts (Fig. S5A). Expression levels of the exocrine genes *Cela2a* (chymotrypsin-like elastase family, member 2A), *Cpa2* (pancreatic carboxypeptidase A2) and *Prss1* (trypsin 1) were also assessed (Fig. S5B). While *Cela2a* mRNA was not detected in reprogrammed cells, and there was no significant increase in *Prss1* expression between control and transduced cells, there was a significant increase in expression of *Cpa2*. However, the overall expression of *Cpa2* in rGBCs was less than 0.001% of that detected in normal pancreas. In order to determine if rGBCs were polyhormonal, insulin-positive GFP+ GBCs were immunostained for somatostatin. Indicative of a polyhormonal phenotype, insulin-positive cells were also somatostatin positive (Fig. S5C). RNA-Seq data from FACS-sorted MIP-GFP+ cells confirmed that insulin-positive cells also expressed *Sst* at levels greater than 20,000 higher than control GBCs ($p < 0.01$).

When PSCs are differentiated toward the pancreatic endocrine fate *in vitro* they are often reported to be capable of tonic insulin secretion, but do not display increased secretion in response to glucose (Kubo et al., 2011; Nostro et al., 2011). In order to determine whether reprogrammed GBCs are superior to PSC derivatives *in vitro*, insulin secretion was measured after exposure to glucose. Similar to cells produced by *in vitro* differentiation of PSCs, rGBCs were able to secrete insulin, as detected using an insulin-specific ELISA (Fig. S6). However, the amount of insulin detected was not significantly different after stimulation with higher glucose concentrations. Hence, like their PSC derived counterparts rGBCs also did not display GSIS, at least at early time points after reprogramming.

## RNA-SEQ analysis revealed only partial reprogramming into mature β-cells

Both *in vitro* differentiated PSCs and the genetically reprogrammed GBCs described here display polyhormonal gene expression and lack of glucose responsiveness. However, the molecular mechanism(s) underlying this imperfect β-cell phenotype are currently unknown. In order to better understand the key differences between rGBCs and true β-cells and to uncover new targets for future reprogramming strategies, we assessed the complete mRNA expression profile of rGBCs. RNA-sequencing technology and bioinformatics were utilized to compare the transcriptomes of adenovirus GFP-only transduced control GBCs, NPM rGBCs (without RA and DBZ), NPM-RA-GSI GBCs (with RA and DBZ) and β-cells. The heatmap depicted in Fig. 4A illustrates that while several groups of genes were expressed in similar fashion between mature β-cells and rGBCs, others were not.

Differential expression analysis between control GBCs, NPM-RA-GSI GBCs and β-cells yielded a large number of differentially expressed genes as shown in Figs. 4B and C. Approximately 1800 genes were up or down regulated with reprogramming after four days. However, a further 3200 to 3400 genes remained differentially expressed between β-cells and rGBCs. A functional analysis of the 1759 genes that were up-regulated in rGBCs indicates that a statistically significant portion was associated with 'neuronal functions', e.g. 'neurotransmission' (p = 1.97E−6) and 'morphology of neurons' (p = 1.7E−1). Since the development of the endocrine pancreas and nervous systems shares many common factors (Atouf et al., 1997), this finding was consistent with the induction of pancreatic endocrine development. However the transcriptional signature of a more mature β-cell phenotype was also observable in that the functions 'quantity of carbohydrate' (p = 1.8E−2; 73 genes) and 'quantity of hormone' (p = 3.8E−3; 69 genes) were present in rGBCs. The most significant canonical pathway was 'MODY signaling' (p = 3.0E−7; 11 genes). Targets of the transcription factors Neurod1, Neurog3, Isl1, Pdx1, Nkx2-2, Pax6, Minx1, and Mafa (p = 8.0E−4) were enriched in this set as well. On the other hand, the most significant functions among the down-regulated genes were related to proliferation, e.g., 'carcinoma' (p = 1.7E−12; 452 genes), 'proliferation of cells' (p = 6.8E−17; 363 genes), and 'cell death' (p = 8.3E−17; 431 genes). 'Immune response' function was also down-regulated (p = 1.1E−8; 227 genes). Targets of the

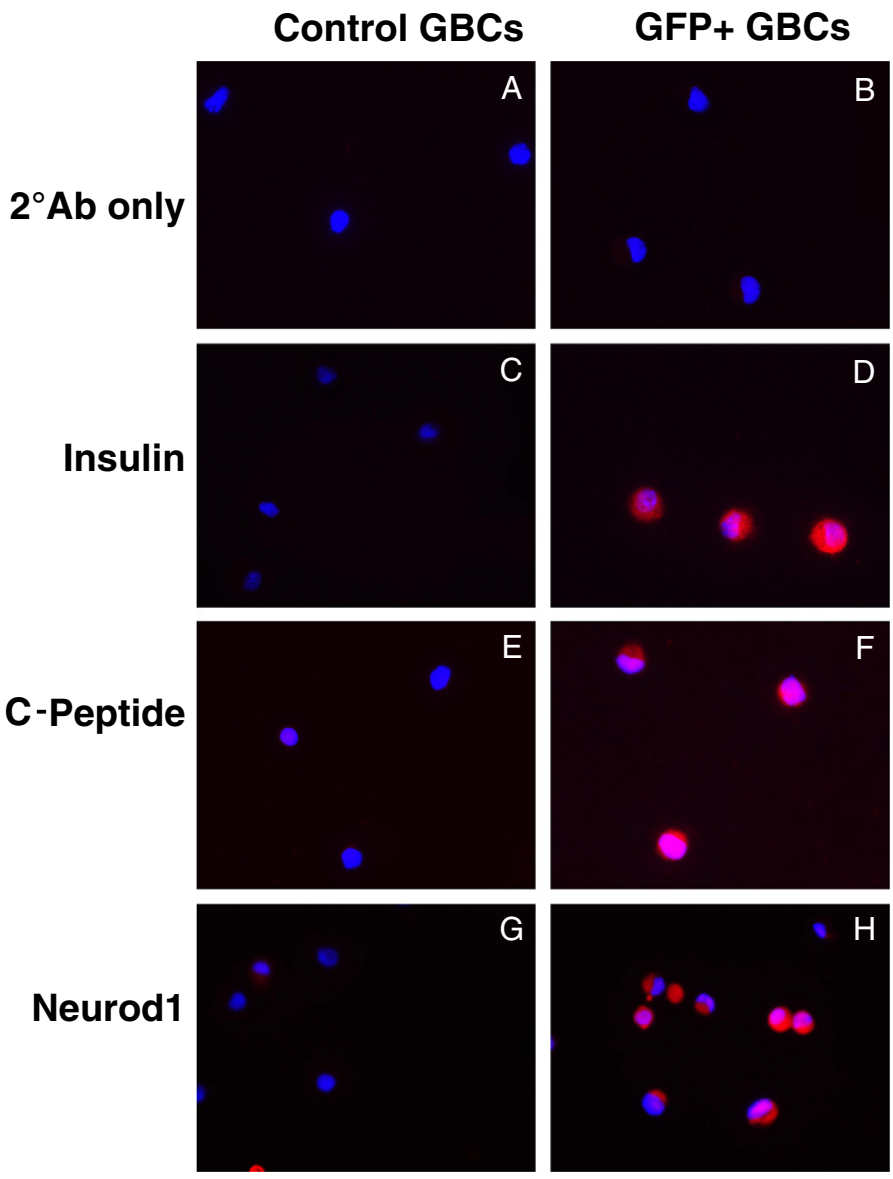


**Figure 3** Characterization of protein expression changes in reprogrammed GBCs. Control or GFP + GBCs were stained with 2° Ab only (A, B) or with antibodies against insulin (C, D), C-peptide (E, F), or Neurod1 (G, H). Representative images are shown for each cell type.

estrogen receptor (ER) (p = 1.0E−8) and transcription factor Hnf4a (p = 2.0E−8) were enriched in this set as well. Despite these signatures suggestive of pancreatic endocrine differentiation, direct comparison of β-cells to rGBCs indicated that many genes related to 'glucose metabolism disorder' (p = 8.2E−4; 204 genes) and 'diabetes mellitus' (p = 9.7E−3; 159 genes) were differentially expressed. These differences are indicative of incomplete reprogramming.

## GBCs are more readily reprogrammed towards the β-cell fate than fibroblasts

Skin fibroblasts are a cell type which is even more readily available than GBCs. Others previously demonstrated that fibroblasts can be differentiated into some functional cell types such as neurons, hepatocyte-like cells, and cardiomyocytes (Huang et al., 2011; Ieda et al., 2010; Vierbuchen et al., 2010). To determine whether skin cells may be a suitable substrate for generating β-cells by reprogramming, tail tip adult mouse fibroblasts from MIP-GFP mice were expanded and exposed to the same reprogramming regimen as GBCs. Three days later, these cells were FACS-sorted based on GFP expression and gene expression analyzed by RT-PCR and qPCR (Figs. 5A–C). Interestingly, expression of *NPM* also was able to induce promoter activity at the *Ins1* locus, as determined by GFP expression and detection of *Ins1* mRNA after three days (Figs. 5A, B). However, other important β-cell transcripts such as *Ins2*, *Neurod1* and *Nkx6-1* were significantly less induced in GFP + fibroblasts than in rGBCs (Fig. 5B). Moreover, other key β-cell transcripts expressed in rGBCs, including *Nkx2-2*, *Pax4* and *Pcsk2*, were not detected at all in GFP + fibroblasts using

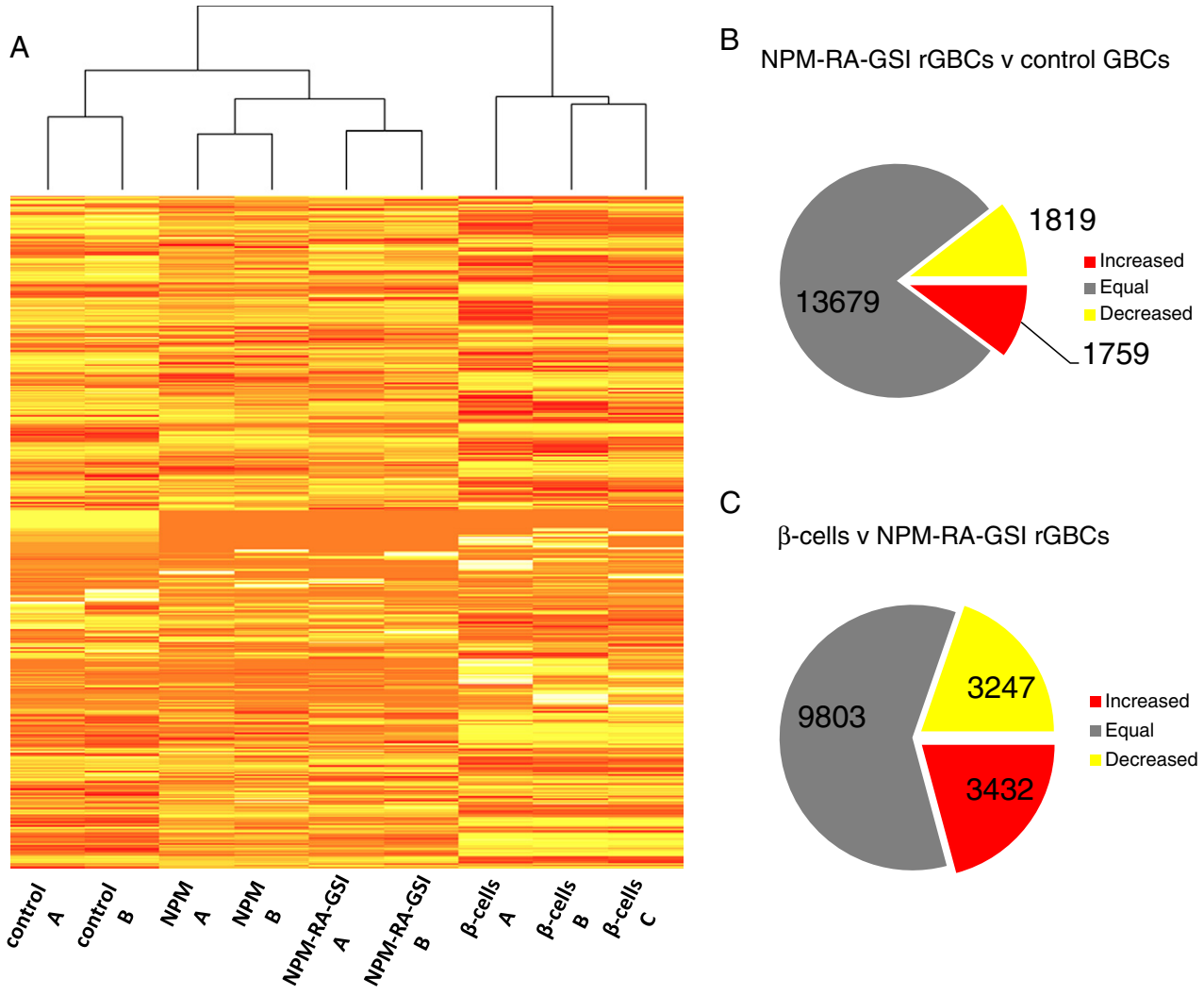


**Figure 4** RNA-Seq analysis of rGBCs. (A) Adenovirus GFP-only infected GBCs (controls A & B) were compared to GBCs reprogrammed with either NPM only (NPM) or with NPM, RA and DBZ (NPM-RA-GSI). Actual pancreatic β-cells (β-cells A & B & C) were used as the optimal target population. The cladogram above the heat-map indicated rGBCs formed an intermediate population of cells with characteristics of both GBCs and β-cells. The number of genes differentially expressed between rGBCs and control GBCs (B) and between β-cells and rGBCs (C) is depicted, showing, for example, that after reprogramming 1759 genes showed increased expression, 1819 genes showed decreased expression, and 13,679 genes were unchanged in rGBCs compared to control GBCs.

RT-PCR (Fig. 5C). Overall, these results indicated that GBCs were clearly more responsive to pancreatic endocrine reprogramming than fibroblasts.

## Transplanted reprogrammed cells can engraft, survive and produce insulin in diabetic mice

Finally, as it has been demonstrated that *in vitro* maturation for several months is needed to generate functional β-cells from PSCs (D'Amour et al., 2006), it was tested whether transplantation of *in vitro* differentiated rGBCs could reverse hyperglycemia in diabetic mice. GBCs reprogrammed with *NPM* and incubated with or without RA were harvested from culture after two to three days and transplanted under the renal capsule of diabetic NOD.Cg-*Rag1*$^{tm1Mom}$ *IL2rg*$^{tm1Wjl}$*Ins2*$^{Akita}$ (NRG-Akita) and NOD.*Cg-Prkdc*$^{scid}$*Il2rg*$^{tm1Wjl}$/*SzJ Ins2*$^{Akita}$ (NSG-Akita) mice (Table S3). Blood glucose levels were monitored weekly. Of the 19 transplanted mice, only one mouse showed a temporary reversal of hyperglycemia that was not sustained (experiment PW0; Fig. S7). Between 8 and 15 weeks after transplantation, all mice were euthanized and the kidneys analyzed by immunohistochemistry by staining with an anti-insulin antibody. Interestingly, although the transplanted cells were unable to permanently reverse the hyperglycemia in any of the recipients, 8/19 of the mice had insulin-positive cells in the graft region of the kidney (Figs. 6A, B) compared with the absence of insulin positive cells detected in any of the control transplanted GBCs (data not shown). To determine if transplanted cells were still polyhormonal, grafts were stained with an antibody against somatostatin (Fig. S8). Some somatostatin-positive cells were also detected in these grafts, but their number was fewer than for insulin-positive cells in the same mice.

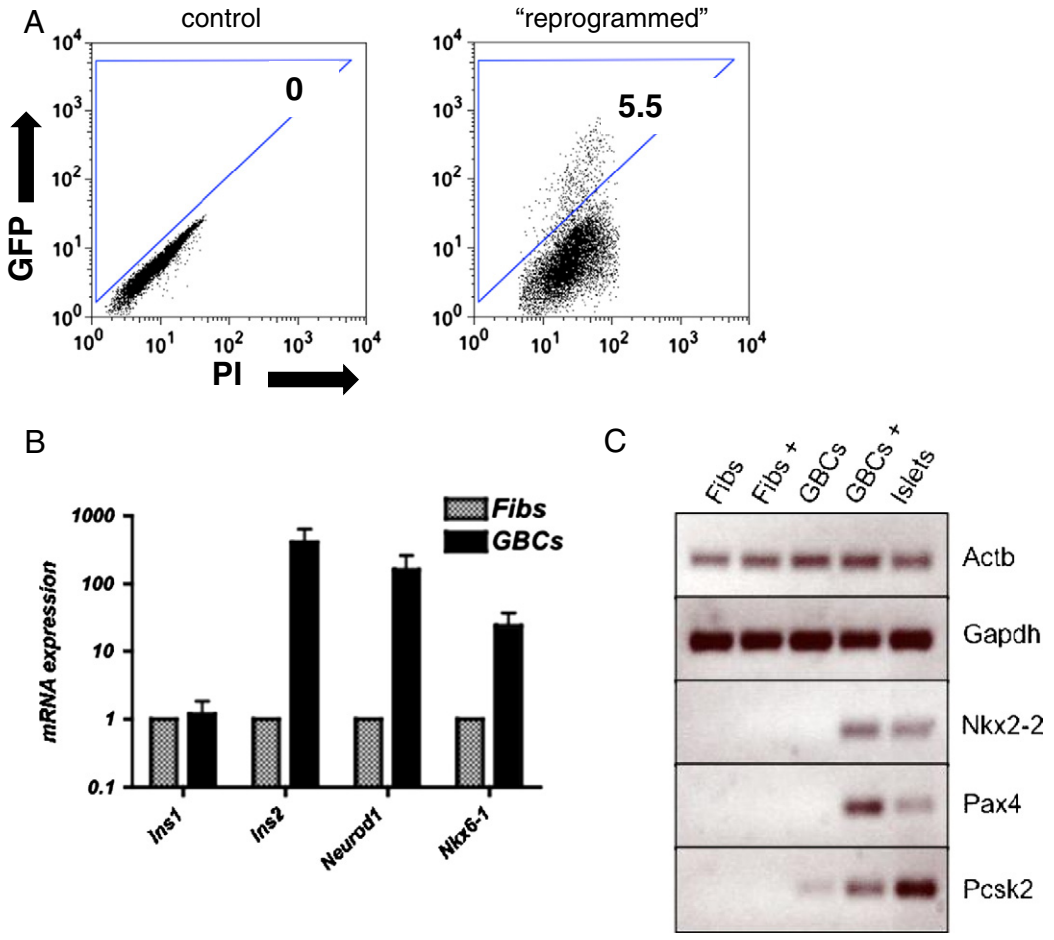


**Figure 5** Attempted reprogramming of fibroblasts to the β-cell fate. (A) By flow cytometric analysis, control MIP-GFP fibroblasts did not contain any GFP+ cells. Adenovirus-NPM-transduced fibroblasts contained 5.5% GFP+ cells three days after reprogramming. (B) FACS-sorted GFP+ fibroblasts expressed *Ins1* mRNA by qPCR. The other β-cell-markers *Ins2*, *Neurod1*, and *Nkx6-1* were expressed at significantly lower levels in GFP+ fibroblasts compared to GFP+ rGBCs (normalized to *Alas1*) ($p < 0.01$; N = 3 replicates for each sample; error bars represent the standard deviation). (C) Using RT-PCR analysis, fibroblasts transduced with NPM (Fibs+) did not express *Nkx2-2*, *Pax4* or *Pcsk2* in contrast to GBCs transduced with NPM (GBC+). *Actb* and *Gapdh* were used as the housekeeping loading controls.

## Discussion

The results presented here establish a combined genetic and small molecule approach to differentiate mouse GBCs towards the pancreatic β-cell lineage (summarized in Fig. S2). The expression of key transcription factors has been shown to be a powerful method to directly reprogram one cell type to another, without the need to go through a pluripotent intermediate (Huang et al., 2011; Ieda et al., 2010; Vierbuchen et al., 2010; Xie et al., 2004). It is interesting that the three transcription factors capable of reprogramming GBCs into islet-like cells are the same as those required for *in vivo* reprogramming of the exocrine pancreas into endocrine pancreas (Zhou et al., 2008), as well as *in vitro* reprogramming of pancreatic exocrine cells into β-like cells (Akinci et al., 2012). Therefore, together these results implicate *Neurog3*, *Pdx1* and *MafA* as the key genetic reprogramming factors needed for the *de novo* generation of mouse β-cells. In addition to expressing these three transcription factors, the frequency of GFP+ rGBCs was augmented by the timed addition of RA and DBZ, a Notch signaling effector. Both these pathways are essential for normal pancreatic development (Apelqvist et al., 1999; Martin et al., 2005; Stafford and Prince, 2002). Manipulation of these pathways has also been used to differentiate pluripotent stem cells towards a differentiated β-cell fate, indicating manipulation of both RA-responsive genes and Notch signaling to also be critical for *de novo* β-cell differentiation (Kroon et al., 2008; Nostro et al., 2011).

Moreover, Notch signaling, particularly involving *Hes1*, has been implicated as a key regulator of cell fate choice during differentiation of the pancreatobiliary progenitor (Spence et al., 2009). In accordance with a shift towards pancreatic differentiation of rGBCs, there was a significant decrease in expression of *Hes1*, supporting a previous study that has shown down-regulation of *Hes1* to be a critical step in differentiation of insulin-positive cells from extrahepatobiliary tissues (Coad et al., 2009). The results presented here further strengthen the case for using GBCs in β-cell reprogramming and expand the previous observations. Firstly, our rGBCs consistently

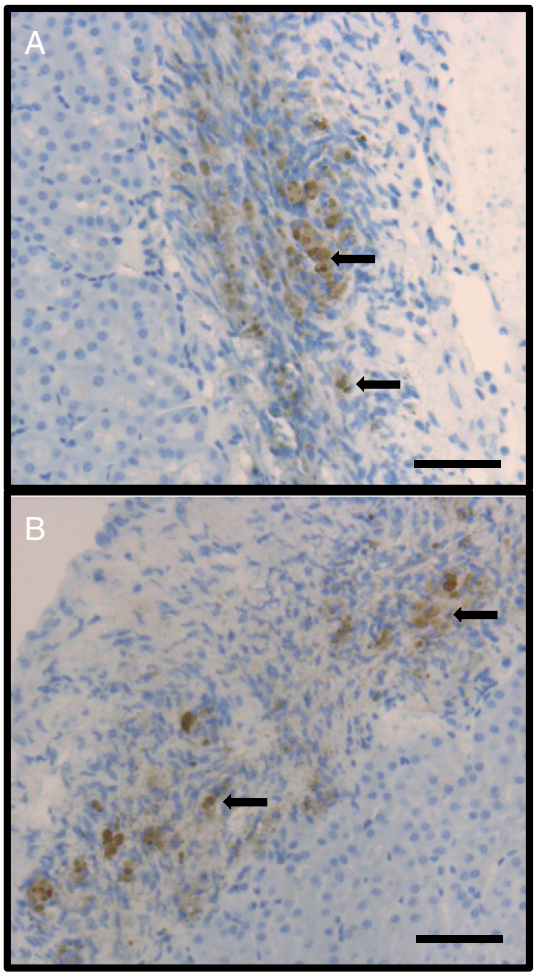


**Figure 6** Immunohistochemistry of transplanted rGBCs. (A, B) Insulin-positive cells (examples marked with black arrows) were detected in 8/19 of the grafts and never in the adjacent kidney. Two representative images are shown here. Scale bars are 100 μm.

express both *Ins1* and *Ins2*, which was not evident in the results of Coad and colleagues (Coad et al., 2009). Moreover, induction of additional key β-cell transcription factors not described in the Coad study, including *Neurod1*, *Nkx2-2* and *Isl1*, was observed here. Additionally, the ability to expand GBCs significantly, while retaining their reprogrammability, was not part of the earlier work.

One of the interesting features of the results presented here is the rapid fate conversion of GBCs into insulin-positive, islet-like cells. Within only 96 h of genetic manipulation by transduction with NPM, de-repression of the insulin loci occurred as evidenced by expression of GFP and detection of both *Ins1* and *Ins2* mRNA. Other gene expression changes also indicated that these cells were differentiating towards the β-cell lineage, including increased expression of the transcription factors *Neurod1*, *Nkx2-2*, *Pax4*, and *Isl1*. In addition, rGBCs increased expression of the proprotein convertases *Pcsk1* and *Pcsk2* which are needed to process the immature proinsulin into insulin and c-peptide. In line with these gene expression changes, processed insulin could be detected by immunocytochemistry and by ELISA in rGBCs. The rapid changes in the gene expression profiles are similar to those observed in other direct reprogramming efforts. For example, Ieda et al. detected alphaMHC-GFP+ cardiomyocytes after only three days of reprogramming from fibroblasts (Ieda et al., 2010). Together with these other studies, the results confirm the power of using key developmental transcription factors for direct reprogramming of various cell types (Huang et al., 2011; Ieda et al., 2010; Vierbuchen et al., 2010; Xie et al., 2004).

It is evident that the cells obtained with the current protocol were not mature, fully functioning β-cells after 96 h. Firstly, the expression of both *Ins1* and *Ins2* mRNA was at a reduced level compared to that seen in pancreatic islets. Secondly, rGBCs were not responsive to glucose stimulation, a common problem for artificially generated β-cells (Kubo et al., 2011; Nostro et al., 2011). Thirdly, the RNA-Seq data showed many genes that were either under-expressed or over-expressed compared to mature adult β-cells. Fourthly, rGBCs were polyhormonal, another common characteristic of *in vitro* reprogrammed cells (D'Amour et al., 2006; Nostro et al., 2011). Finally, rGBCs were unable to reverse the hyperglycemic state of diabetic mice after transplantation. However, it is possible that the engrafted cells were simply too few in numbers to have a substantial effect on blood glucose levels. In fact, insulin-positive cells could be detected in the grafts of transplanted mice. Alternatively, some form of *in vivo* maturation may be required for generation of functioning β-cells, as has been shown to be needed for the transplantation of ESC-derived β-cells into diabetic mice (Kroon et al., 2008). Further studies are required to investigate the *in vivo* maturation of transplanted rGBCs.

A common phenotype of most *in vitro*-derived β-like cells is the absence of GSIS (Kubo et al., 2011; Nostro et al., 2011). Similar to these studies, we could not reprogram GBCs to acquire GSIS, an essential feature of typical pancreatic β-cells. In the future, therefore, it will be imperative to determine the missing factor(s) required to instigate glucose sensing, insulin packaging and insulin secretion. The comparison of rGBCs to β-cells from type 2 diabetic patients may be appropriate. Type 2 diabetic patients exhibit defective insulin secretion, exemplified by the loss of GSIS. We analyzed the expression of known genes that when mutated can result in loss of insulin secretion. One factor that was under-expressed in rGBCs relative to β-cells was transmembrane protein 27, encoded by *Tmem27*. Also known as Collectrin, this factor controls insulin exocytosis and has been implicated as a key component of glucose-stimulated insulin secretion (Fukui et al., 2005). Decreased *Tmem27* gene expression has also been detected in islets from type 2 diabetic patients (Altirriba et al., 2010). Other genes that were not expressed at comparable levels to actual β-cells were *Slc30a8* and *Nnat* that are involved in vesicle maturation and insulin secretion respectively (Artner et al., 2010). Future reprogramming studies should be aimed at the induction of these genes that are essential for mature β-cell function.

In conclusion, the data presented here support a novel strategy to generate pancreatic β-like cells. The gall bladder is a readily accessible and non-essential tissue that contains cells amenable to large-scale expansion and reprogramming to a pancreatic fate. In addition, our data indicate that this endodermal derivative is more amenable to reprogramming than skin fibroblasts. However, the reprogramming is currently only partial and the rGBCs did not become fully functional,

mature β-cells *in vitro*. Although there were many up-regulated marker genes detected in rGBCs, global RNA-Seq analysis was needed to properly characterize the cells relative to true functional β-cells, something that is commonly missing in other reprogramming studies. Nonetheless, the results outlined here will be useful for further experiments aimed at generating a direct differentiation-based cell therapy for type 1 diabetes in human patients.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.02.005.

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